

**Supplementary Information:**

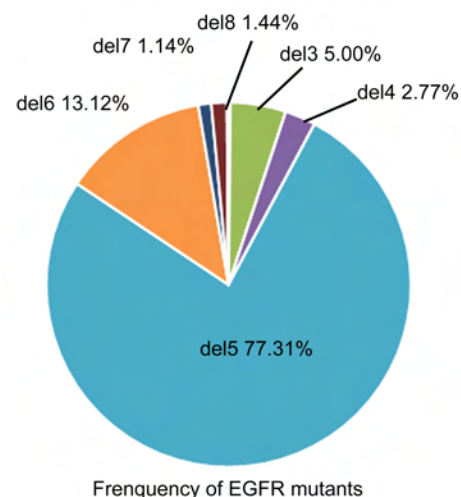
**Table S1. The Conserved Motifs/Residues of RAF Family Kinases Regulating Their Activity**

Raf Isoform	NtA motif	Catalytic spine mutation	Regulatory spine mutation	Central RH alteration in dimer interface	APE motif
ARAF	<sup>299</sup> SGYY <sub>302</sub>	V324F	L358M	R362H	<sup>474</sup> AAE <sub>476</sub>
BRAF	<sup>446</sup> SSDD <sub>449</sub>	V471F	L505M	R509H	<sup>621</sup> APE <sub>623</sub>
CRAF	<sup>338</sup> SSYY <sub>341</sub>	V363F	L397M	R401H	<sup>513</sup> APE <sub>515</sub>

**Table S1. The Conserved Motifs/Residues of RAF Family Kinases Regulating Their Activity.**  
The key motifs/residues of RAF family kinases involved in this study were listed in this table.

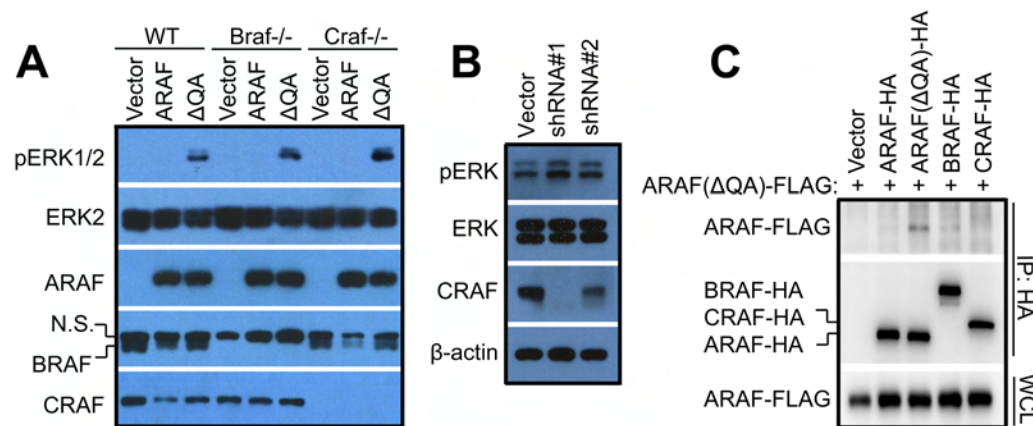
54 Table S2. Protein Kinase Mutants With In-frame Deletions of  $\beta 3$ - $\alpha C$  Loop in Cancers

	$\beta 3$	$\alpha C$	No.		$\beta 3$	$\alpha C$	No.
ARAF	VAVKVLKVSQPTAEQAQAFKNEQVLRKTR			EGFR	VAIKELREAT-SPKANKEILDEAYVMASVD		
	VAVKVLKVSQPTAE--QAFKNEQVLRKTR		1		VAIKEPT-----SPKANKEILDEAYVMASVD		1
BRAF	VAVKMLNVTAPTQQQLQAFKNEVGVLRLKTR				VAIKE-----PKANKEILDEAYVMASVD		97
	VAVK---VTAPTQQQLQAFKNEVGVLRLKTR		1		VAIKEX-----PKANKEILDEAYVMASVD		7
	VAVKM-----TPQQQLQAFKNEVGVLRLKTR		1		VAIKEXX-----PKANKEILDEAYVMASVD		4
	VAVKMY-----TPQQQLQAFKNEVGVLRLKTR		1		VAIKE-----KANKEILDEAYVMASVD		33
	VAVKMF-----TPQQQLQAFKNEVGVLRLKTR		1		VAIKEX-----KANKEILDEAYVMASVD		321
	VAVKML-----TPQQQLQAFKNEVGVLRLKTR		6		VAIKECG-----KANKEILDEAYVMASVD		1
	VAVKML-----PQQQLQAFKNEVGVLRLKTR		1		VAIKE-----ANKEILDEAYVMASVD		3
	VAVKMLK-----PQQQLQAFKNEVGVLRLKTR		1		VAIKEN-----ANKEILDEAYVMASVD		1
	VAVKMLN-----PQQQLQAFKNEVGVLRLKTR		2		VAIKEXX-----ANKEILDEAYVMASVD		2
	VAVKMLNA-----QQLQAFKNEVGVLRLKTR		1		VAIKEANKG-----ANKEILDEAYVMASVD		1
	VAVKMLNV-----QQLQAFKNEVGVLRLKTR		2		VAIKE-----NKEILDEAYVMASVD		1
	VAVKMLNVK-----QLQAFKNEVGVLRLKTR		2		VAIKEXX-----NKEILDEAYVMASVD		3
	VAVKMLNVT-----LQAFKNEVGVLRLKTR		2		VAIKESKG-----NKEILDEAYVMASVD		5
JAK1	VILKVLDP-----HRDISLAFFEAASMMRQV				VAIKESKDD-----KEILDEAYVMASVD		1
DomainB	VILKLV-----DISLAFFEAASMMRQV		2		VAIKEL-----PKANKEILDEAYVMASVD		1
	VILKVLDP-----H-----ISLAFFEAASMMRQV		2		VAIKEL-----KANKEILDEAYVMASVD		2
	VILKVLDP-----HRDISLA-FFEAASMMRQV		1		VAIKELRQ-----KANKEILDEAYVMASVD		1
ERBB2	VAIKVLRENT-SPKANKEILDEAYVMAGVG				VAIKELRQP-----EILDEAYVMASVD		2
	VAIKV-----SPKANKEILDEAYVMAGVG		1		VAIKELRE-----ANKEILDEAYVMASVD		2
	VAIKVA-----PKANKEILDEAYVMAGVG		1		VAIKELRE-----EILDEAYVMASVD		2
EGFR	VAIKELREAT-SPKANKEILDEAYVMASVD				VAIKELRE-----ILDEAYVMASVD		2
	VALKR-----AT-SPKANKEILDEAYVMASVD		1		VAIKELREP-----ILDEAYVMASVD		3
	VAVK-----T-SPKANKEILDEAYVMASVD		1		VAIKELREXX-----LDEAYVMASVD		2
	VAI-----REAT-SPKANKEILDEAYVMASVD		1		VAIKELREA-----ILDEAYVMASVD		5
	VAI-----AT-SPKANKEILDEAYVMASVD		14		VAIKELREA-----ILDEAYVMASVD		1
	VAI-----T-SPKANKEILDEAYVMASVD		19		VAIKELREA-----LDEAYVMASVD		2
	VAIT-----T-SPKANKEILDEAYVMASVD		4		VAIKELREAX-----LDEAYVMASVD		22
	VAIK-LREAT-SPKANKEILDEAYVMASVD		2		VAIKELREAA-C-----LDEAYVMASVD		1
	VAIK-----EAT-SPKANKEILDEAYVMASVD		2		VAIKELREAX-XX-----LDEAYVMASVD		3
	VAIK-----AT-SPKANKEILDEAYVMASVD		13		VAIKELREAT-----LDEAYVMASVD		27
	VAIKY-----AT-SPKANKEILDEAYVMASVD		1		VAIKELREAT-----D		1
	VAIK-----T-SPKANKEILDEAYVMASVD		3489		VAIKELREAT-S-----LDEAYVMASVD		1
	VAIKX-----T-SPKANKEILDEAYVMASVD		6		VAIKELREAT-SP-----LDEAYVMASVD		1
	VAIKXX-----T-SPKANKEILDEAYVMASVD		24		VAIKELREAT-SPKA-----DEAYVMASVD		1
	VAIK-----SPKANKEILDEAYVMASVD		29				
	VAIKX-----SPKANKEILDEAYVMASVD		79				
	VAIKXX-----SPKANKEILDEAYVMASVD		24				
	VAIK-----PKANKEILDEAYVMASVD		16				
	VAIKX-----PKANKEILDEAYVMASVD		152				
	VAIKIV-----PKANKEILDEAYVMASVD		2				
	VAIK-----KANKEILDEAYVMASVD		5				
	VAIKXX-----KANKEILDEAYVMASVD		13				
	VAIK-----NKEILDEAYVMASVD		1				
	VAIKE-REAT-SPKANKEILDEAYVMASVD		3				
	VAIKE--EAT-SPKANKEILDEAYVMASVD		1				
	VAIKE--AT-SPKANKEILDEAYVMASVD		28				
	VAIKEX--AT-SPKANKEILDEAYVMASVD		2				
	VAIKE--T-SPKANKEILDEAYVMASVD		14				
	VAIKEX--T-SPKANKEILDEAYVMASVD		184				
	VAIKE-----SPKANKEILDEAYVMASVD		199				
	VAIKEX-----SPKANKEILDEAYVMASVD		73				



55 Table S2. Protein Kinase Mutants With In-frame Deletions of  $\beta 3$ - $\alpha C$  Loop in Cancer. To explore  
56 all protein kinase mutants with variable  $\beta 3$ - $\alpha C$  loop deletions in cancer genomes, we we interrogated  
57 the ICGC (International Cancer Genome Consortium) database, the cBioportal for Cancer Genomics  
58 database, and the COSMIC (Catalogue of Somatic Mutations in Cancer) database. All known  
59 mutations including those reported in literatures<sup>1-4</sup> were summarized in this table.  
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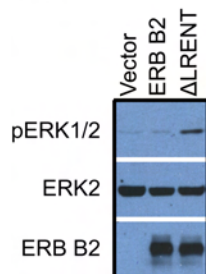
## Figure S1



**Figure S1 (related to Figure 1). ARAF mutant with Q347A348 deletion is activated by elevated homodimerization but not heterodimerization.**

A, ARAF(ΔQA) and its wild-type counterpart were stably expressed in RAF-knockout fibroblasts, and their activity was measured by anti-phosphoERK1/2 immunoblot. ARAF(ΔQA) activates MEK-ERK signaling in wild-type, BRAF<sup>-/-</sup>, and CRAF<sup>-/-</sup> MEFs. B, the CRAF knockdown does not affect the activity of ARAF(ΔQA) in BRAF<sup>-/-</sup> MEFs. The activity of ARAF(ΔQA) in BRAF<sup>-/-</sup> with or without shRNAs against CRAF was measured as phosphoERK by immunoblot. C, ARAF(ΔQA) hardly heterodimerizes with wild-type ARAF, BRAF and CRAF. The wild-type RAF molecules were coexpressed with ARAF(ΔQA) in 293T cells, and the coimmunoprecipitation assay was carried out as described before<sup>5</sup>. All images are representative of at least three independent experiments.

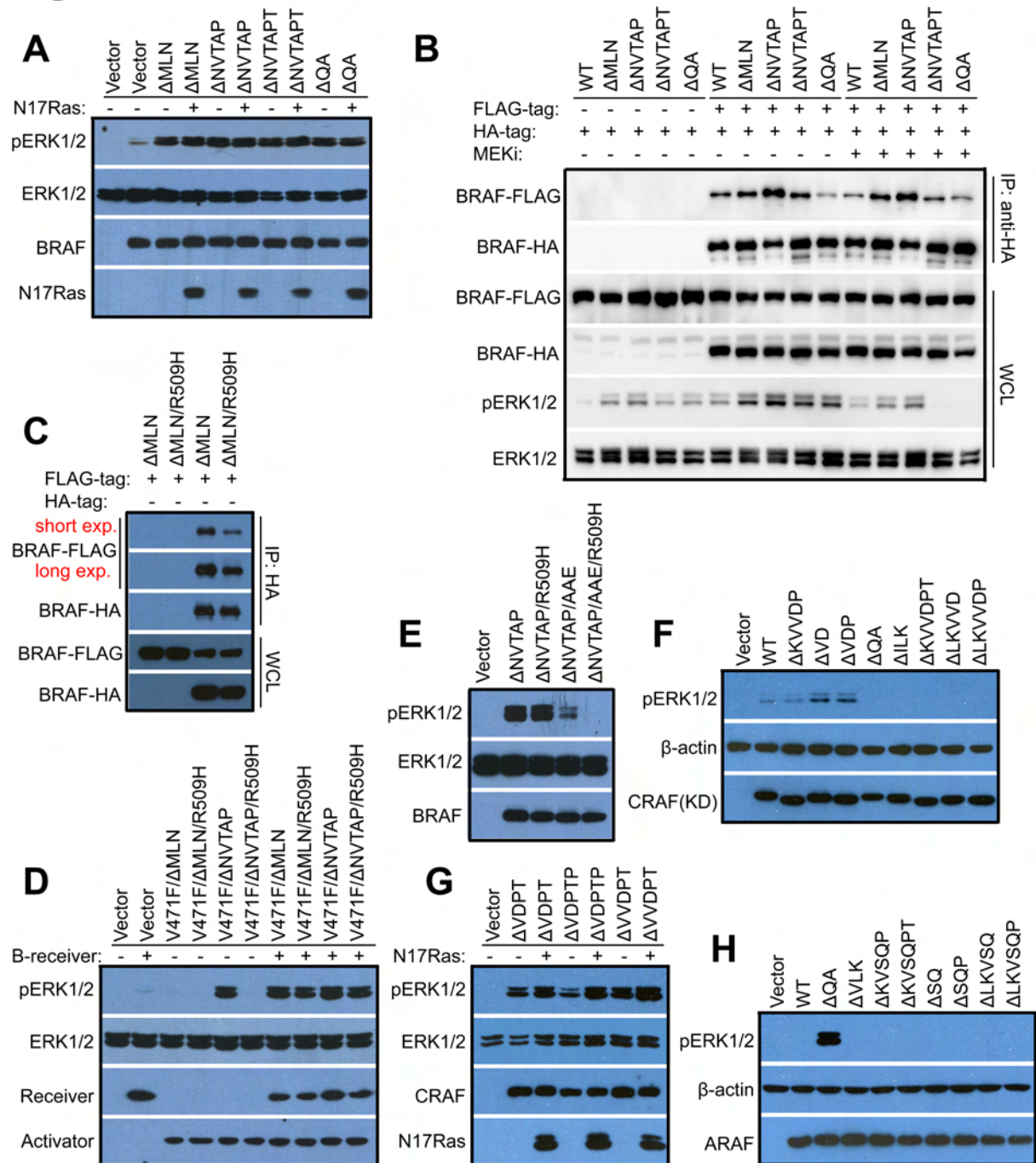
## Figure S2



**Figure S2. An ERB B2 mutant with β3-αC loop deletion has constitutive activity.**

The ERB B2 mutant with in-frame β3-αC loop deletion was expressed in 293T cells and its activity was measured by anti-phosphoERK1/2 immunoblot. All images are representative of at least three independent experiments.

## Figure S3



**Figure S3 (related to Figure 4). RAF kinase is activated by in-frame β3-αC loop deletions through enhanced homodimerization.**

(A) BRAF mutants with in-frame β3-αC loop deletions exhibit a RAS-independent activity. The BRAF mutants were expressed with or without N17RAS in 293T cells and their activity was measured by anti-phosphoERK1/2 immunoblot.

(B-C) BRAF mutants with in-frame β3-αC loop deletions have increased albeit variable dimer affinity. The dimer affinity of BRAF mutants in 293T transfectants treated with or without MEK inhibitor was measured by co-immunoprecipitation as in Figure 1E.

(D) The central R509H alteration in dimer interface is not able to abolish the allosteric activity of Catalytic spine-fused BRAF mutants with a high dimer affinity toward BRAF-receiver. The RAF co-activation assay was carried out as described before<sup>5,6</sup>.



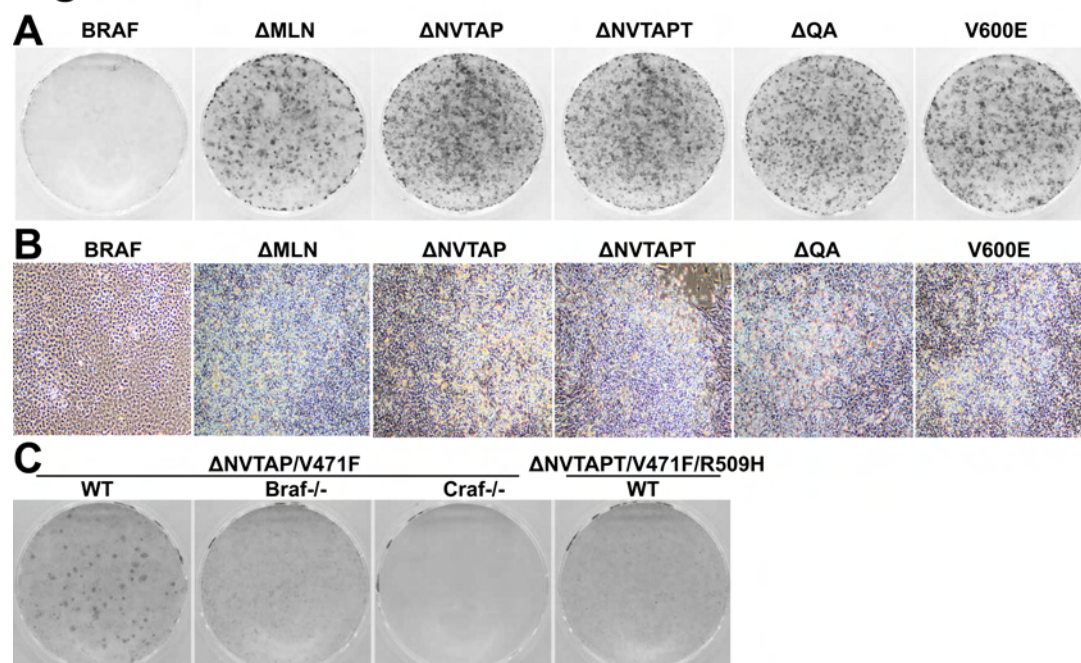
(E) The combined alteration of APE motif and the central Arg in dimer interface abolishes the activity of BRAF mutant with a high dimer affinity. The BRAF mutants were expressed in 293T cells and their activity was measured by anti-phosphoERK1/2 immunoblot.

(F-G) In-frame  $\beta 3$ - $\alpha C$  loop deletions turn on CRAF independent of RAS. The activity of CRAF mutants expressed in 293T cell with or without N17RAS was measured as in (A).

(H) ARAF is activated by  $\Delta QA$  but not other deletions in  $\beta 3$ - $\alpha C$  loop. The activity of ARAF mutants expressed in 293T cells was measured as in (A).

All images are representative of at least three independent experiments.

## Figure S4



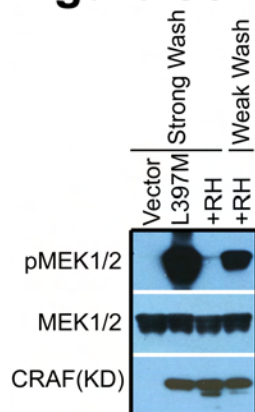
**Figure S4 (related to Figure 5). BRAF mutants with in-frame  $\beta 3$ - $\alpha C$  loop deletions have a strong oncogenic potential.**

(A-B) BRAF mutants with in-frame  $\beta 3$ - $\alpha C$  loop deletions transform both BRAF-knockout and CRAF-knockout immortalized fibroblasts. A, foci formation assay of immortalized *braf*<sup>-/-</sup> MEFs that express BRAF mutants. B, cellular morphology images of immortalized *craf*<sup>-/-</sup> MEFs that express BRAF mutants.

(C) The transforming ability of kinase-dead BRAF( $\Delta NVTAP/V471F$ ) depends on endogenous RAF molecules and dimerization. Foci formation assay of immortalized MEFs that express BRAF mutant was carried out as in Figure 1C.

All images are representative of at least three independent experiments.

## Figure S5

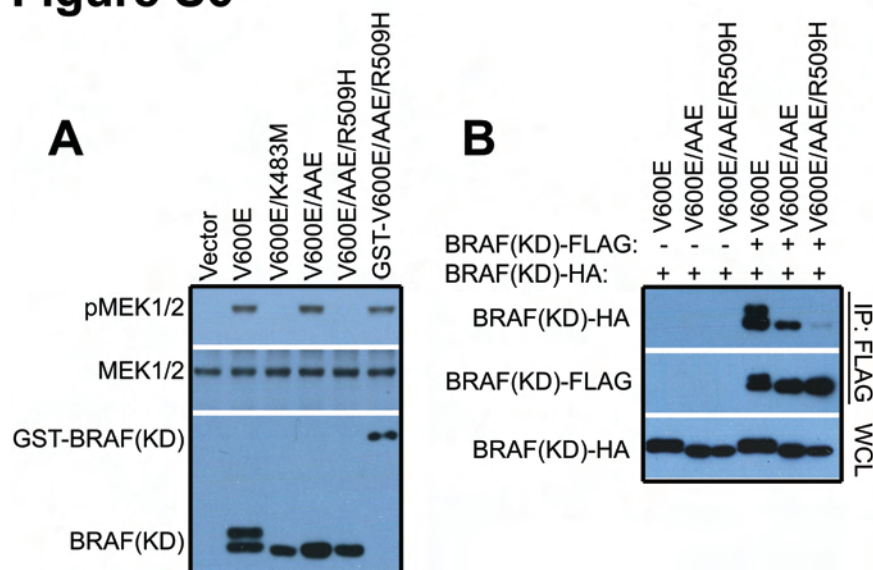


**Figure S5 (related to Figure 6). The constitutively active CRAF R-spine mutant with a low dimer affinity retains partial catalytic activity in vitro upon purification with a gentle wash of PBS.**

CRAF mutants were expressed in 293T cells and purified by immunoprecipitation with either a strong wash of RIPA buffer (1% NP-40) or a gentle wash of PBS. Then their activity was measured by in vitro kinase assay as in Figure 6.

All images are representative of at least three independent experiments.

## Figure S6



**Figure S6 (related to Figure 7). The BRAF(V600E) mutant with non-canonical APE motif has constitutive activity but is sensitive to RH alteration in dimer interface.**

(A) The BRAF(V600E) mutant with non-canonical APE motif can phosphorylate MEK as well as its prototype, which is blocked by further RH alteration in dimer interface. BRAF mutants were expressed in 293T cells and the phospho-MEK1/2 was detected by immunoblot. The kinase-dead BRAF(V600E) mutant, BRAF(V600E/K483M) was used as control.

(B) The non-canonical APE motif decreases the dimer affinity of BRAF(V600E) but can not completely abolish its dimerization. FLAG- and HA-tagged BRAF mutants were co-expressed in 293T cells that were lysed with RIPA buffer containing 0.1% NP-40, and the co-immunoprecipitation assay was carried out with a gentle wash of PBS as in Figure S5.

All images are representative of at least three independent experiments.

## Supplementary Materials and Methods

To knockdown CRAF in *braf*<sup>-/-</sup> MEFs that stably express ARAF( $\Delta$ QA), shRNAs were designed by using a website software (<http://katahdin.cshl.org/siRNA/RNAi.cgi?type=shRNA>), and the lentiviral vectors were constructed by using traditional molecular cloning methods. The targeting sequences were listed as follow:

shRNA#1 5- GGAATGGAATGAGCTTACA-3

shRNA#2 5- GGAATGAGCTTACATGACT-3

## Reference

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5. Hu J, Stites EC, Yu H, Germino EA, Meharena HS, Stork PJ *et al.* Allosteric activation of functionally asymmetric RAF kinase dimers. *Cell* 2013; 154: 1036-1046.
6. Hu J, Ahuja LG, Meharena HS, Kannan N, Kornev AP, Taylor SS *et al.* Kinase regulation by hydrophobic spine assembly in cancer. *Mol Cell Biol* 2015; 35: 264-276.